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Coprinopsis neophlyctidospora sp. nov., a new ammonia fungus from boreal forests in Canada

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Abstract *Coprinopsis neophlyctidospora* sp. nov. (*Basidiomycota, Agaricales*), collected in urea treated soil of boreal forests from Canada is described and illustrated. Its micromorphological features, phylogenetic analysis, and mating test delineate this taxon as a new species. In addition, its ecological characters also indicate it is a new ammonia fungus.

Key words biogeography, cryptic species, ITS, species-complex, taxonomy

Introduction

The ammonia fungi are defined as a chemoecological group of fungi whose growth can be stimulated by the addition of urea or aqueous ammonia or any other related nitrogenous materials to forest soils (Sagara 1975). The diversity and ecology of ammonia fungi have been studied in diverse geographical areas such as in Japan, Taiwan, New Zealand, Western Australia, UK and western US (Suzuki et al. 2003). However, ammonia fungi in the boreal region of North America had not previously been examined. We, therefore, conducted the first survey of ammonia fungi in boreal forests of Canada. In this study, we focused on an ammonia fungus in the *Coprinopsis phlyctidospora* species-complex. *Coprinopsis phlyctidospora* had been believed to be a cosmopolitan species (Fukiharu & Horigome 1996, Suzuki et al. 2003), though we have revealed that this group represents of cryptic species (Suzuki et al. 2002) in different areas of the world. In this study, we reveal morphological characters and phylogenetic relationships based on rDNA nucleotide sequences of a new

saprobic *Coprinopsis* ammonia fungus collected from the urea treated soil of boreal forests in Canada.

Materials & methods

Isolation and culture

A mixture litter (A_0) and soil from the upper A horizon from a lodge pole pine (*Pinus contorta* var. *latifolia*) forest and from an aspen (*Populus tremuloides*) forest both near Edmonton, Canada was collected in May 2001. To stimulate fruiting of ammonia fungi, a large amount of aqueous urea (granular fertilizer; 46% nitrogen, 5–20 mg urea/g dry soil) was applied in plant pots containing the soil/litter mixture from two different forests separately and incubated at 23–25°C under a 12 hrs light/12 hrs dark regime for 30–72 days. Sterile water was applied at 1–3 day intervals to avoid desiccation of the surface of the mixture. Basidiomata were collected from plant pots and cultured in the laboratory. All cultures were grown at 25°C under a 12 hrs light/12 hrs dark regime on MY agar medium [(malt extract 10 g/L (Difco, USA), yeast extract 2g/L (Difco, USA) and agar 15g/L (Nakalai, Japan)].

Morphology

All descriptions of macro- and microscopic features were obtained from cultivated basidiomata. Different developmental stages of basidiomata were photographed and collected to observe the changes in pileus colors and veil features. Anatomical observations and measurements were made on material mounted in 25% aqueous ammonia. Microscopic terminology of basidiospores and cystidia followed that of Vellinga (1988). Color terms and notations used in this description are based on Kornerup & Wanscher (1978). Herbarium abbreviations are according to Holmgren & Holmgren (1998). Basidiospore statistics: x_m, the arithmetic mean of spore length by spore width (± standard deviation) ; n, number of spores measured; Q, the quotient of spore length and spore width; Q_m, the mean of Q-values (± standard deviation). For scanning electron microscope (SEM) observation of basidiospores samples from spore prints were rehydrated in 25% aqueous ammonia and fixed in 2.5% osmic acid, coated with platinum-palladium in an ion sputter-coater (Hitachi E-1030; Hitachi Tokyo, Japan), and observed under a SEM (Hitachi S-800) operating at 15.0 kV.

Phylogeny

Fungal strains were grown in a MY liquid medium. Mycelia were harvested, squeezed with a paper towel, frozen and lyophilized. The dried mycelia were then ground with a spatula and suspended in a TES buffer [50 mM Tris-HCl (pH 7.5), 20mM EDTA, 1% SDS] and soluble fractions were recovered by centrifugation. The DNA was purified by a TE buffer [10 mM Tris-HCl (pH 8.0), 1mM EDTA] saturated phenol/chloroform/isoamyl alcohol (Nippon gene) extraction followed by an iso-propyl alcohol precipitation. After desiccation of the DNA pellet, the DNA was dissolved in 30 µl TE buffer. For some samples, the genomic DNA was further purified using a NucleoSpin Extract II (Macherey-Nagel) following the manufacture's recommendation. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGTATGC-3') were used to amplify the ITS1-5.8S-ITS4 ribosomal DNA region. PCR reactions were carried out using Ex Taq (TakaraBio) according to the manufacturer's protocol. PCR

Table 1: *Coprinopsis* collections included in the phylogenetic analysis.

Taxon	Isolate no.	Voucher specimen no.	Locality	Dominating vegetation	GenBank accession no.
Coprinopsis neophlyctidospora	CHU2009	UAMH11230*	Nojack, Alberta, CAN	*Pinus contorta* var. *latifolia*	AB564409
	CHU2021	CBM FB33899*	Chipman, Alberta, CAN	*Populus tremuloides*	AB564407
	CHU2022	CBM FB33901*	Chipman, Alberta, CAN	*P. tremuloides*	AB564406
	CHU2023	CBM FB33894*	Chipman, Alberta, CAN	*P. tremuloides*	AB564408
	CHU2024	CBM FB33896*	Chipman, Alberta, CAN	*P. tremuloides*	AB564410
	CHU2025	CBM FB38024*	Chipman, Alberta, CAN	*P. tremuloides*	AB564721
GenBank sequences					
C. phlyctidospora △	NBRC30478**		Kyoto, Japan	*Castanopsis cuspidata*	AB071615
	CHU3010	CBM FB24544**	Chiba, Japan	*Quercus acuta*, *C. cuspidata*	AB071614
	CHU3017	CBM FB224548**	Chiba, Japan	*Q. acuta*, *C. cuspidata*	AB071616
	CHU3003	CBM FB24539*	Tokyo, Japan	*Q. acuta*	AB071609
		CBM FB21061*	Tokyo, Japan	*Q. acuta*	AB071610
C. "austrophlyctidospora" △	CHU3014	CBM FB29564*	North Island, NZ	*Nothofagus menziesii*, *N. fusca*	AB071793
	CHU3015	CBM FB29560*	North Island, NZ	*N. menziesii* , *N. fusca*	AB071794
	CHU3016	CBM FB30247*	North Island, NZ	*N. menziesii* , *N. fusca*	AB071795
	CHU3007**		North Island, NZ	*Pinus radiata* (Plantation)	AB071789
C. echinospora △		CBM FB21264*	Hokkaido, Japan	*Fagus crenata*	AB071798
		CBM FB21629*	Aomori, Japan	*F. crenata*	AB071799
		CBM FB21725*	Aomori, Japan	*F. crenata*	AB071800
		CBM FB21733*	Miyagi, Japan	*F. crenata*	AB071801
	CHU3019*		Kyoto, Japan	*C. cuspidata*	AB071803
Outgroup					
C. atramentaria	KACC49358				AF345814

* Basidiomata obtained by urea treatment in laboratory experiment; ** Basidiomata obtained by urea treatment in field experiment

△ Reference, Suzuki et al. 2002

products were purified using the NucleoSpin Extract II, and DNA fragments were directly sequenced using the BigDye Terminator ver3.1 Cycle Sequencing Kit (Applied Biosystems) according to the provided protocol. The reactions were then cleaned up using the Centri Sep (Princeton Separations) before analysis by capillary electrophoresis on a 3130x DNA Analyzer (Applied Biosystems). Sequences were assembled and edited using the ATSQ software (Genetyx). All nucleotide sequences were deposited in GenBank/EMBL/DDJB and accession numbers are provided (Table 1). The data set was aligned using Clustal X ver. 1.81 (Jeannmougin et al. 1998), and the resulting alignment was manually refined. The alignment was deposited in TreeBASE under accession number http://purl.org/phylo/treebase/phylows/study/TB2:S10848. All phylogenetic analyses were performed with Paup* 4.0b10 (Swofford 2001). Gaps were treated as missing data. The ITS data set of 730 bp was analysed with maximum parsimony. The maximum parsimony was performed with a full heuristic search with 100 random stepwise addition replicates and TBR (tree bisection-reconnection) branch swapping and equal weighting of all characters. The robustness of inferred MP tree topologies was tested by the bootstrap value (Felsenstein 1985) with 1000 replicates. *Coprinopsis atramentaria* was used as an outgroup. Sequences for *C. atramentaria, C. phlyctidospora, C. "austrophlyctidospora"* and *C. echinospora* were retrieved from GenBank (Table 1).

Mating tests

Mating compatibility of *C. neophlyctidospora* with *C. phlyctidospora* and *C. "austrophlyctidospora"* was examined. Monokaryotic testers of *C. phlyctidospora* NBRC 30478 (Suzuki et al. 2002) and *C. "austrophlyctidospora"* CHU 3007 (tester strains were generated by the authors of this paper, unpublished) were used in this study. Small mycelium-covered agar blocks of dikaryotic *C. neophlyctidospora* and one of the monokaryotic testers were placed 5 mm apart in the center of an MY agar plate. After about two weeks incubation at 25°C in the dark, several pieces of mycelia from the outer edge of the monokaryotic colony were examined and compatible crossings were determined by the presence of clamp connections (Tables 2, 3). Individual pairings were performed three times.

Taxonomy

Coprinopsis neophlyctidospora Raut, Fukiharu & A. Suzuki, **sp. nov.** Figs. 1–3

MycoBank 518507

Pileo primo 5–12 mm lato 3–6 mm alto, usque ad 10–30 mm lato, ovato-campanulato, demum in margine lacerato revolutoque, radiatim sulcato, candido vel eburneo, deinde cinerascenti, primo squamis albis radiatim hirsuto-fibrillosis ubique tecto, dein fere glabro; carne tenuissima, fragilissima, alba, sapore miti, odore nullo; lamellis adnexis vel liberis, comfertis, angustis (0.5–1.0 mm), albis, deinde atratis, deliquescentibus; stipete 50–80 mm longo, 1–3 mm crasso, aequali vel sursum leviter attenuato, basi leviter incrassatula, cavo, candido, fragilissimo, primo squamis albis fibrillosis squarrosis ubique tecto, dein nudo; basidiosporis in cumulo atratis, sub microscopio rufo-brunneis, 7.8–8.5 µm longis × 5.4–6.0 µm anticis × 5.4–5.8 latis, ovoideis vel ellipsoideis, verrcosis, poro germinationis centrali 1.8–2.1 µm lato, cum plagiis; basidiis 26.2–36.2 × 7.7–8.5µm, tetrasporis; pleurocystidiis 40–65 × 15–30 µm, obovatis vel utriculiformibus, hyalinis, tenui-tunicatis; cheilocystidiis 25–35 × 15–25 µm, subglobosis vel utriculiformibus, hyalinis, tenui-tunicatis; velo pilei,

Fig. 1. Macromorphology of the newly described species, *Coprinopsis neophlyctidospora*. A. Basidiomata (UAMH 11230); B. Longitudinal section of basidioma (UAMH 11230); and C E different developmental stages of basidiomata. Bars, A B 5 mm, C E 20 mm.

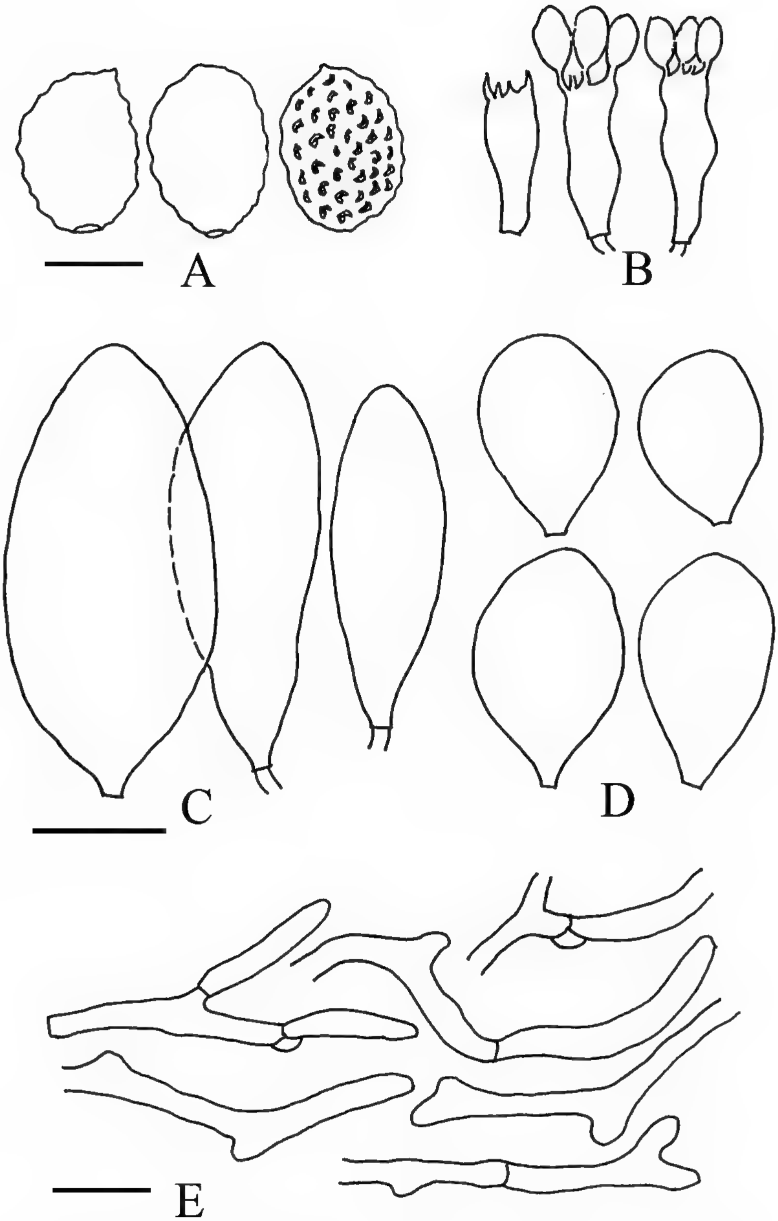

Fig. 2. Anatomical details of *Coprinopsis neophlyctidospora* (drawn from the holotype)
A. Basidiospores B. Basidia C. Pleurocystidia D. Cheilocystidia D. Veil elements.
Bars A 5 µm B–D 20 µm, E 30 µm.

ex hyphis multi-septatis, divaricatis, tenui-tunicatis, hyalinis, 28.2 68.2 × 5.8 7.8 μm compositis; fiblis praesentibus.

Type: Canada, Alberta: Nojack (115°35′15"W 53°36′19"N; 828 m) on a large amount of aqueous urea (granular fertilizer; 46% nitrogen, 5 mg urea/g dry soil) applied soil/litter mixture collected from pine (*Pinus contorta* var. *latifolia*) forest, May 2001, A. Suzuki [CHU 2009] (Holotype UAMH 11230).

Etymology: The Latin *neo-* refers to the "New World" origin of the species, and -*phlyctidospora* reflects its morphological resemblance to *Coprinopsis phlyctidospora*.

Pileus 5–12 mm broad, 3–6 mm high in button stage, when young ellipsoid to ovoid, later convex to plane, 10–30 mm broad when expanded, finally uplifted with age, radially sulcate; pileipellis color at first grayish orange to pale orange (5B, 3), soon becoming grayish brown (5D-E, 3), surface when young densely covered with white, hairy fibrillose scales (Fig.1), soon breaking up in small radially arranged fibrillose veil, later almost glabrous or veil remaining only in the center. Context very thin, fragile, white; taste mild; odor indistinct. Lamellae adnexed to free, close (number of lamellae 40–60), with 0–1 lamellulae (between two lamellae), narrow (0.5–1.0 mm wide), edge slightly pruinose, at first white, then grayish, finally blackish, deliquescent. Stipe up to 50–80 × 1–3 mm, central, cylindrical, equal or somewhat tapering upward, sometimes the base clavate, not rooting, fistulose, fragile, surface white, at first with white fibrillose scales (Fig. 1), soon becoming smooth (Fig. 1). Basidiospores black in mass, dark reddish brown under the light microscope, 7.8–8.5 × 5.4–6.0 μm in face view (x_m = 7.9 ± 0.6 × 5.8 ± 0.5 μm, Q = 1.2–1.5, Q_m = 1.4 ± 0.1, n = 40), 5.4–5.8 μm diam in side view (x_m = 5.6 ± 0.4 μm, n = 40), ovoid to ellipsoid with warty ornamentation, a central germ pore 1.8–2.1 (1.8 ± 0.1, n = 10) μm wide and vague plage (Figs. 2, 3A). Basidia 26.2–36.2 × 7.7–8.5 μm, 4-spored (Fig. 3C). Pleurocystidia 40–65 × 15–30 μm, obovoid or clavate to utriform, thin-walled, colorless (Fig. 3C). Cheilocystidia 25–35 × 15–25 μm, subglobose or obovoid to utriform, numerous, thin-walled, colorless (Fig. 3D). Elements of veil on the pileal surface composed of thin-walled, diverticulate, colorless hyphae, 28.2–68.2 × 5.8–7.8 μm (Fig. 3E). Clamp connections present in vegetative mycelia and in pileal veil elements.

Habit & Habitat – Saprobic, growing after application of urea in the soil collected from lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) and aspen (*Populus tremuloides* Michx.) forests in Canada. Fruiting of this fungus in urea-treated soil indicates that it is an ammonia fungus (Sagara 1975).

Distribution – Only known from the type locality in Canada.

Additional specimens examined: CANADA. alberta: Chipman, (112°45′10"W 53°39′46"N; 728 m) in a large amount of aqueous urea (granular fertilizer; 46% nitrogen, 20 mg urea/g dry soil) applied soil/litter mixture collected from an aspen (*Populus tremuloides*) forest, CBM-FB33899, CBM-FB33901, CBM-FB33894, CBM-FB33896 December 2003, T. Fukiharu, CBM-FB38024 January 2004, T. Fukiharu.

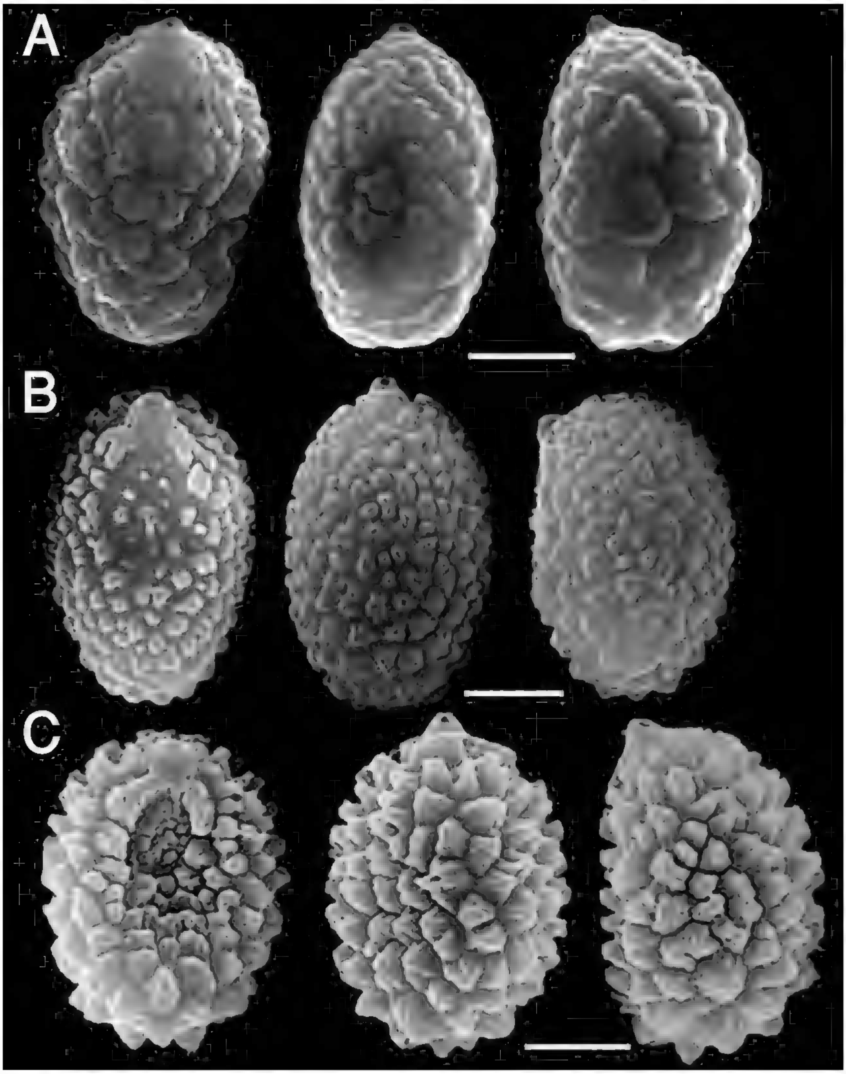

Fig. 3. Comparison of scanning electron micrographs (SEM) of basidiospores among: A. *Coprinopsis neophlyctidospora;* B. *C. "austrophlyctidospora"* (Fukiharu et al. 2011); and C. *C. phlyctidospora* (Fukiharu et al. 2011). Bars, A–C = 2.5 µm.

Results & discussion

According to conventional *Coprinus* Pers. taxonomy, *Coprinopsis neophlyctidospora* belongs to *Coprinus* subsect. *Alachuani* (Singer 1986) based on its diverticulate veil elements. In this subsection four species are reported

to have warty ornamented basidiospores, i.e., *Coprinopsis echinospora* (Buller) Redhead & al. (≡ *Coprinus echinosporus*), *Coprinopsis phlyctidospora* (Romagn.) Redhead & al. (≡ *Coprinus phlyctidosporus*), *Coprinopsis rugosobispora* (J. Geesink & Imler) Redhead & al. (≡ *Coprinus rugosobisporus*) (Orton & Watling 1979, Moser 1983, Uljé & Noordeloos 1997, Uljé 2005), and *Coprinopsis "austrophlyctidospora"* nom. prov. (proposed as a new species by Fukiharu et al. 2011). Macroscopically it is difficult to separate *C. neophlyctidospora* from these species, but some microscopic features are distinctive. *Coprinopsis echinospora* differs in its amygdaliform basidiospores and two hyphal types in the pileal veil. *Coprinopsis rugosobispora* has two-spored basidia and is also distinguished from *C. phlyctidospora* and *C. "austrophlyctidospora"*

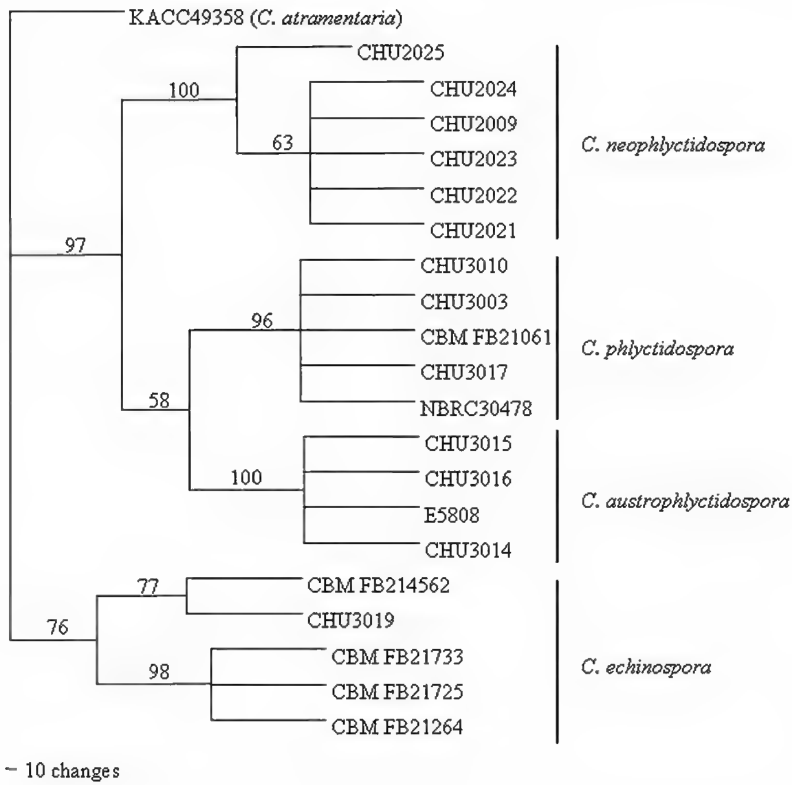

FIG. 4. The most parsimonious tree (156 steps) from a heuristic search based on the nuclear rDNA sequences in ITS region for phylogenetically related *Coprinopsis* spp. The tree was rooted to the outgroup, *C. atramentaria*. The numbers on branches indicate bootstrap support (value >50% only shown) with 1000 replications. The distance corresponding to 10 base changes per 1000 nucleotide position is indicated by the bar. Sidebar represents the inferred clades of *Coprinopsis* species.

TABLE 2: Dikaryon-monokaryon mating reactions between the dikaryotic isolates of *Coprinopsis neophlyctidospora* from Canada and monokaryotic testers of *C. phlyctidospora* (NBRC30478).

Dikaryotic Isolates	Monokaryotic testers of *C. phlyctidospora* from Japan							
	A_1B_1		A_2B_2		A_1B_2		A_2B_1	
	CHU003	CHU001	CHU008	CHU007	CHU010	CHU009	CHU011	CHU012
CHU2009								
CHU2021								
CHU2022								
CHU2023								

, Clamp connections did not form

TABLE 3: Dikaryon-monokaryon mating reactions between the dikaryotic isolates of *Coprinopsis neophlyctidospora* from Canada and monokaryotic testers of *C. "austrophlyctidospora"* (CHU3007).

Dikaryotic isolates	Monokaryotic testers of *C. "austrophlyctidospora"* from New Zealand							
	A_1B_1		A_2B_2		A_1B_2		A_2B_1	
	CHU013	CHU015	CHU021	CHU022	CHU016	CHU020	CHU018	CHU019
CHU2009								
CHU2021								
CHU2022								
CHU2023								

, Clamp connections did not form

TABLE 4: Diagnostic basidiospore features of *Coprinopsis neophlyctidospora* and its allies.

BASIDIOSPORE CHARACTER	*C. neophlyctidospora*	*C. phlyctidospora*	*C. "austrophlyctidospora"*
Size (µm)	7.8 8.5 × 5.4 6.0	8.4 10.6 × 6.0 7.6	6.5 7.5 × 5.1 6.5
Surface (verrucose)	warts more flattened, plage not distinct	warts coarse & cone like, plage +/ distinct	warts smaller, plage always distinct

in basidiospore size and surface ornamentation. The basidiospore size of *C. neophlyctidospora* is smaller than that of *C. phlyctidospora* (8.4–10.6 × 6.0–7.6 µm; Uljé and Noordeloos 1997, Uljé 2005) while larger and more elongated than those of *C. "austrophlyctidospora"* (6.5–7.5 × 5.1–6.5 µm; Fukiharu et al. 2011). The scanning electron micrographs also revealed its distinct broadly warted basidiospores, showing warts more flattened than those in other species

(Fig. 2). Furthermore, in the phylogenetic analysis based on ITS nucleotide sequences *C. neophlyctidospora* formed a conspicuous and distinct clade among its allies. The tree topology is strongly supported by high bootstrap values (Fig. 3), indicating that *C. neophlyctidospora* is a distinct taxon. Moreover, the mating test results comparing *C. neophlyctidospora* with *C. phlyctidospora* (Table 2) and *C. "austrophlyctidospora"* (Table 3) also strongly support biological separation of these species. In conclusion, we propose *C. neophlyctidospora* as a new cryptic species within the *C. phlyctidospora* species-complex, distributed in the boreal region of Canada. Further research in unexplored regions is needed to resolve comprehensive global understanding of the distribution of these species.

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